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Ultrasonic enhancement of antibiotic action on several species of bacteria

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The effect of the antibiotics gentamicin, streptomycin, kanamycin, tetracycline, and ampicillin on planktonic cultures of *Enterobacter aerogenes*, *Serratia marcescens*, *Salmonella derby*, *Streptococcus mitis*, and *Staphylococcus epidermidis* with and without an application of 70 kHz ultrasound was studied. The ultrasound was applied at levels that had no inhibitory effect on planktonic cultures of bacteria. Measurements of viability at, above, and below the minimum inhibitory concentration of the above antibiotics on the planktonic cultures of these bacteria showed that a simultaneous application of 70 kHz ultrasound and antibiotic significantly increased the effectiveness of selected antibiotics. Bacterial viability was reduced several orders of magnitude when harmless levels of ultrasound were combined with some antibiotics, especially the aminoglycosides. Similar synergistic effects of combined ultrasound and antibiotic treatment were seen in both Gram-positive and Gram-negative bacteria with several classes of antibiotics. These results may have application in the treatment of bacterial infections normally resistant to some antibiotics.

Key Words——antibiotics; bioacoustic effect; *E. aerogenes*; *S. derby*; *S. epidermidis*; *S. marcescens*; *S mitis*; ultrasound

Previous work has shown that gentamicin treatment of *Pseudomonas aeruginosa* and *Escherichia coli* coupled with ultrasound (Hedges et al., 1980; Komrakov and Antipov, 1990; Kondo et al., 1989; Scherba et al., 1991; Williams, 1983) enhances the bactericidal activity of the antibiotic against planktonic and sessile bacteria (Pitt et al., 1994; Qian, 1996; Qian et al., 1996, 1997; Williams and Pitt, 1997). The present research has extended this type of treatment to other antibiotics and to other species of bacteria. Its purpose was to conduct a screening study in which a variety of representative Gram-positive and Gram-negative organisms were studied. The antibiotics selected for this study represent several different agents effective against the organisms selected. Specifically, we will report the ultrasonic enhancement of gentamicin, kanamycin, streptomycin, tetracycline, and ampicillin on *Enterobacter aerogenes*, *Serratia marcescens*, *Salmonella derby*, *Streptococcus mitis*, and *Staphylococcus epidermidis*. In this screening study, not all antibiotics were tested with each organism.

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Materials and Methods

Organisms. Cultures of *E. aerogenes* (ATCC 13048), *S. marcescens* (ATCC 8100), *S. derby* (API 245143), *S. epidermidis* (ATCC 14460), and *S. mitis* (PI 519) were maintained on Columbia agar plates. These species of bacteria were chosen because they represent several types of Gram-positive and Gram-negative bacteria. Twenty-four hours before an experiment, 10 ml of either tryptic soy broth (TSB) without glucose (Difco, Detroit, MI) or Todd Hewitt broth (THB, Difco) was inoculated from a plate and grown overnight at 37°C. After 24 h, the culture was diluted 1 : 1,000 into sterile TSB or THB and grown at 37°C in 50-ml Erlenmeyer flasks on a rotary shaker at 150 rpm. Growth times for the second culture varied and were based on when the individual species reached the exponential growth phase. The number of bacteria in the suspensions was measured by serial dilutions in physiological saline solution (PSS) and plating onto

nutrient or Columbia agar. Plates were incubated for 24 h at 37°C.

Antibiotics. Gentamicin sulfate, kanamycin monosulfate, streptomycin sulfate, tetracycline hydrochloride, and ampicillin (D[−]-α-Aminobenzylpenicillin) were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Using sterile distilled water, gentamicin was diluted to 40 μg/ml, kanamycin to 50 μg/ml, streptomycin to 50 μg/ml, tetracycline to 100 μg/ml, and ampicillin to 100 μg/ml.

Ultrasonication. Ultrasonic exposure was generated with a Sonicor SC-100 sonicating bath (Sonicor Instrument Co., Copiaque, NY) operating at 70 kHz and a power density of about 3 W/cm^2, as measured by a calibrated hydrophone (Bruel and Kjaer, Naerum, Denmark). The ultrasonic power was generated by two lead zirconate crystals connected to the underside surface of a stainless steel bath. The bath was filled with approximately 1,500 ml of distilled water and maintained at a constant temperature of 37°C by recirculating the water through a temperature control unit (Refrigeration Circulator, Cole-Parmer, Vernon Hills, IL, USA).

Measurements of MIC. The minimum inhibitory concentration (MIC) of the different antibiotics was determined as reported previously (Pitt et al., 1994). Briefly, the MIC was measured by preparing a series of test tubes containing TSB and the antibiotic at increasing concentrations from 0 to 100 μg/ml, depending on the antibiotic used. Each tube was inoculated with a dilute culture of exponential-growth-phase organisms and incubated at 37°C for 24 h, after which the turbidities of the cultures were assessed. The MIC was defined as the lowest concentration of antibiotic that inhibited turbidity in the test tube.

Measurement of bactericidal activity. Four sets of duplicate test tubes were prepared for each organism with or without an antibiotic. The first set contained 1 ml of a culture of bacteria grown in TSB or THB for 24 h on a rotary shaker at 150 rpm, diluted in 1 ml of sterile distilled water. This set was incubated at 37°C. The second set contained 1 ml of a culture of bacteria, diluted in 1 ml of distilled water, and was placed in the Sonicor bath at 37°C. Ultrasonication was continuous throughout the experiment. The third set contained a dilute culture of bacteria with a concentration of antibiotic at the MIC of the antibiotic for that organism, and it was incubated at 37°C, similar to the first set. The fourth set contained a dilute culture of organisms with the same antibiotic concentration as the first set and was placed in the Sonicor bath at 37°C, with sonication throughout the experiment. Samples were taken from each of the tubes at selected time points, serially diluted in PSS, and 100 μl were inoculated onto petri dishes containing either nutrient agar or Columbia agar (used for *S. derby* which required a richer growth medium) by using the spread plate method. The petri dishes containing the serially diluted organisms were incubated at 37°C for 24 h and counted. The mean and 95% confidence intervals of the log of the counts of colony forming units (cfu) per ml were calculated from the results of several replicate experiments. Samples were taken from each of the test tubes at 0-, 1-, 2-, and 3-h time intervals with concentrations of antibiotic at the MIC. If a strong synergistic bioacoustic effect was noted at the MIC, several concentrations of antibiotic below the MIC were tested. A "strong synergistic bioacoustic effect" was defined as more than 1 log of killing by ultrasound and antibiotic over killing by antibiotic alone. For example, concentrations of antibiotic at 67 and 33% of the MIC were tested on *S. marcescens* with gentamicin. If no bioacoustic effect was seen at the MIC, the procedure was modified to use higher concentrations of antibiotic with sampling times at 0-, 1-, 2-, 3-, and 6-h time intervals. Higher concentrations of antibiotic were used in the following experiments: *E. aerogenes* treated with gentamicin and tetracycline and *S. epidermidis* treated with ampicillin. All other experiments followed the standard procedure above.

Statistical analysis. The model used to describe and analyze the changes in log concentration data was a repeated measures model, an appropriate model because multiple measurements were taken over time on each test tube. Variables in the model included antibiotic concentration, time, set, and sample. The model also involved all two-way and three-way interactions between set, treatment, and time. These interactions made the model nonadditive, meaning that the difference in log concentrations between each treatment was not constant across the different sampling times. Therefore the differences in treatments were evaluated at each level of time. Furthermore, the errors were determined to be correlated, and thus a correlated error structure (first-order autoregression model) was used to analyze the data.

Results

Gram-negative organisms

E. aerogenes treated with gentamicin. The MIC of gentamicin for *E. aerogenes* was determined to be 8 μg/ml. The results of ultrasonic experiments show that ultrasound alone did not decrease *E. aerogenes* viability. Gentamicin at the MIC decreased viability by 1 log after 3 h, and gentamicin in combination with ultrasound decreased viability by 4 logs after 3 h, 3 logs greater than antibiotic alone (see Fig. 1). The *p*-value for the log of the difference between antibiotic alone vs. antibiotic with sonication was 0.0002 after 3 h. This

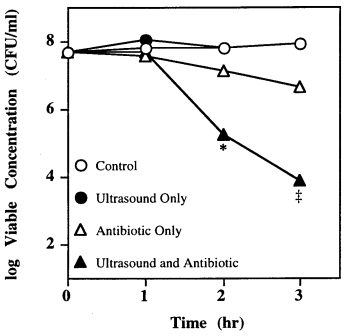

Fig. 1. Viability of a 3-h culture of *E. aerogenes* after exposure to combinations of gentamicin and ultrasound.

Open symbols represent no ultrasonic exposure; closed symbols represent insonation at 3 W/cm^2 at 70 kHz. ○, 0 μg/ml; △, 8 μg/ml of gentamicin. Statistically significant differences between samples exposed to antibiotics and ultrasound and those exposed only to antibiotics are noted: * ($p<0.05$) or ‡ ($p<0.001$).

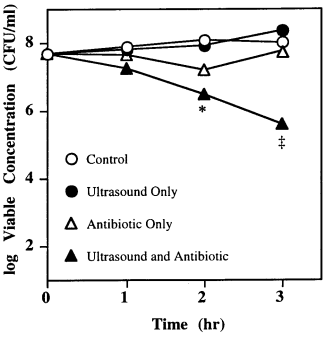

Fig. 2. Viability of a 3-h culture of *E. aerogenes* after exposure to combinations of kanamycin and ultrasound.

Open symbols represent no ultrasonic exposure; closed symbols represent insonation at 3 W/cm^2 at 70 kHz. ○, 0 μg/ml; △, 14 μg/ml of kanamycin. Statistically significant differences between samples exposed to antibiotics and ultrasound and those exposed only to antibiotics are noted: * ($p<0.05$) or ‡ ($p<0.001$).

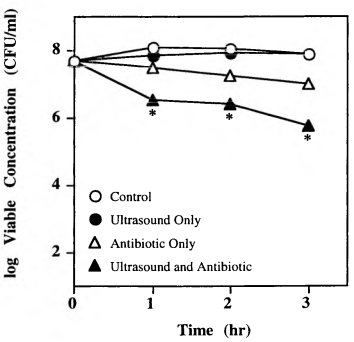

Fig. 3. Viability of a 3-h culture of *E. aerogenes* after exposure to combinations of streptomycin and ultrasound.

Open symbols represent no ultrasonic exposure; closed symbols represent insonation at 3 W/cm^2 at 70 kHz. ○, 0 μg/ml; △, 14 μg/ml of streptomycin. Statistically significant differences between samples exposed to antibiotics and ultrasound and those exposed only to antibiotics are noted: * ($p<0.05$).

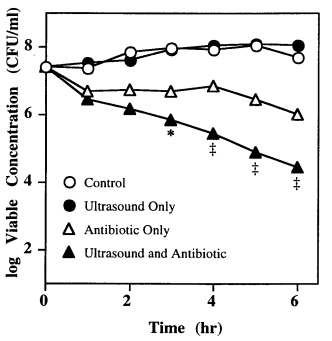

Fig. 4. Viability of a 3-h culture of *E. aerogenes* after exposure to combinations of tetracycline and ultrasound.

Open symbols represent no ultrasonic exposure; closed symbols represent insonation at 3 W/cm^2 at 70 kHz. ○, 0 μg/ml; △, 50 μg/ml of tetracycline. Statistically significant differences between samples exposed to antibiotics and ultrasound and those exposed only to antibiotics are noted: * ($p<0.05$) or ‡ ($p<0.001$).

bioacoustic effect was first evident after 2 h of exposure. Experiments performed at 20 and 10 μg/ml showed that gentamicin in combination with ultrasound killed so effectively that no viable cfu were found after 1 or 2 h of exposure (data not shown).

E. aerogenes treated with kanamycin. The MIC of kanamycin for *E. aerogenes* was determined to be 14 μg/ml. Similar to the previous experiment, these data show that ultrasound alone does not decrease *E. aerogenes* viability. Kanamycin at the MIC did not decrease viability, but kanamycin in combination with ultrasound decreased viability by 2 logs after 3 h (see Fig. 2; $p<0.0004$ after 3 h). Some enhanced killing was seen after 1 h.

E. aerogenes treated with streptomycin. The MIC of streptomycin for *E. aerogenes* was also determined to be 14 μg/ml. The same experimental procedure outlined above was followed for *E. aerogenes* with streptomycin at 14 μg/ml. *E. aerogenes* appeared to be more resistant to this antibiotic than the others did and showed only 1 log more killing with ultrasound and antibiotic than with antibiotic alone after 3 h of exposure (see Fig. 3; $p<0.0028$ after 3 h). The enhanced killing was statistically significant at all time points.

E. aerogenes treated with tetracycline. The MIC of tetracycline for *E. aerogenes* was determined to be 19 μg/ml. Experiments were performed at and above the MIC. There was no significant effect of the antibiotic combined with ultrasound at or above the MIC (at 19 and 30 μg/ml, respectively; data not shown). However, after 4 h of ultrasonication at 50 μg/ml of antibiotic (more than double the MIC), a significant synergistic effect of ultrasound and antibiotic was seen (see Fig. 4; $p<0.0006$ after 6 h). The combination of ultrasound and antibiotic showed enhanced bactericidal activity, producing greater than 2 logs more killing than antibiotic alone after 6 h of exposure.

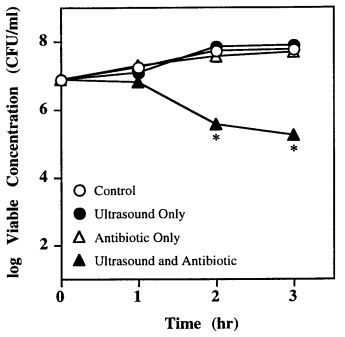

Fig. 5. Viability of a 3-h culture of *S. marcescens* after exposure to combinations of gentamicin and ultrasound.

Open symbols represent no ultrasonic exposure; closed symbols represent insonation at 3 W/cm^2 at 70 kHz. ○, 0 μg/ml; △, 2 μg/ml of gentamicin. Statistically significant differences between samples exposed to antibiotics and ultrasound and those exposed only to antibiotics are noted: * ($p<0.05$).

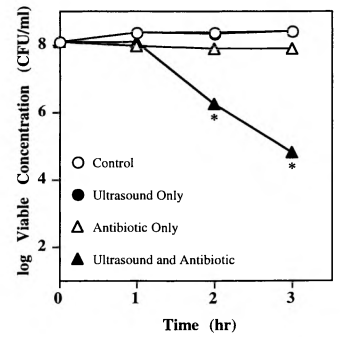

Fig. 6. Viability of a 6-h culture of *S. derby* after exposure to combinations of gentamicin and ultrasound.

Open symbols represent no ultrasonic exposure; closed symbols represent insonation at 3 W/cm^2 at 70 kHz. ○, 0 μg/ml; △, 30 μg/ml of gentamicin. Statistically significant differences between samples exposed to antibiotics and ultrasound and those exposed only to antibiotics are noted: * ($p<0.05$).

S. marcescens treated with gentamicin. The MIC of gentamicin for *S. marcescens* was determined to be 6 μg/ml. Ultrasonic enhanced killing was evident at the MIC and at 66 and 33% of the MIC (4 and 2 μg/ml, respectively). As with *E. aerogenes*, ultrasound alone had no effect on the viability of *S. marcescens*. Experiments run at the MIC showed killing with ultrasound was 3 logs greater than with antibiotic alone after 3 h (data not shown). The experiments at 66 and 33% of the MIC were performed to determine if the ultrasonic enhancement could be extended to levels below the MIC. These results were also positive. At 33% of the MIC, the experiments showed more than 3 logs of killing with ultrasound combined with antibiotic above killing with antibiotic alone after 3 h of exposure (see Fig. 5; $p<0.0001$ after 3 h).

S. marcescens treated with tetracycline. The MIC was determined to be 19 μg/ml for tetracycline on *S. marcescens*. The results of these experiments were inconclusive because no statistically significant killing by antibiotic alone or by antibiotic with ultrasound was seen at 19 or 30 μg/ml after 3 h (data not shown).

S. derby treated with gentamicin. The MIC was determined to be 11 μg/ml for gentamicin on *S. derby*. Ultrasound alone had no effect on the viability of *S. derby*. Experiments using ultrasound and gentamicin at the MIC showed killing 1 to 2 logs greater than with gentamicin alone after 3 h of exposure (data not shown). Experiments with ultrasound at more than twice the MIC (30 μg/ml of gentamicin) showed killing 3 to 4 logs greater than with antibiotic alone after 3 h of exposure (see Fig. 6; $p<0.0012$ after 3 h).

S. derby treated with tetracycline. The MIC of tetracycline for *S. derby* was determined to be 5 μg/ml. Experiments at and above the MIC (up to four times the MIC) showed no bioacoustic effect. These data are not shown.

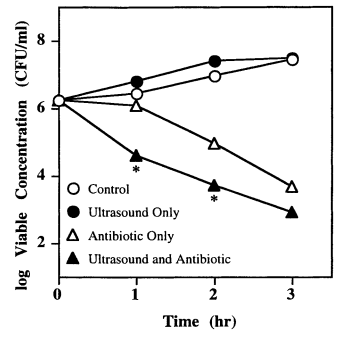

Fig. 7. Viability of a 3-h culture of *S. mitis* after exposure to combinations of ampicillin and ultrasound.

Open symbols represent no ultrasonic exposure; closed symbols represent insonation at 3 W/cm^2 at 70 kHz. ○, 0 μg/ml; △, 3 μg/ml of ampicillin. Statistically significant differences between samples exposed to antibiotics and ultrasound and those exposed only to antibiotics are noted: * ($p<0.05$).

Gram-positive organisms

S. mitis treated with ampicillin. The MIC was determined to be 3 μg/ml for ampicillin on *S. mitis*. Ultrasound alone had no effect on the viability of *S. mitis*. The bioacoustic effect could be noted after the first hour. Even though the bactericidal effect of the antibiotic was not greatly different than the effect of the combined antibiotic and ultrasound after 3 h, a slight synergistic effect because of ultrasound was still noted, especially at shorter exposure times (see Fig. 7; $p<0.0030$ after 1 h, $p<0.1729$ after 3 h).

S. epidermidis treated with ampicillin. The MIC for *S. epidermidis* with ampicillin was difficult to determine because of sporadic positive tubes. Therefore experiments were performed at a concentration known to be bactericidal: 75 μg/ml of antibiotic. The procedure was modified so that samples were taken at 0-, 1-, 2-, 3-, and 6-h time intervals. At these intervals, a synergistic effect between the ultrasound and ampicillin was

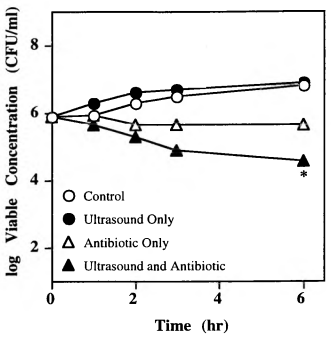

Fig. 8. Viability of a 3-h culture of *S. epidermidis* after exposure to combinations of ampicillin and ultrasound.

Open symbols represent no ultrasonic exposure; closed symbols represent insonation at 3 W/cm^2 at 70 kHz. ○, 0 μg/ml; △, 75 μg/ml of ampicillin. Statistically significant differences between samples exposed to antibiotics and ultrasound and those exposed only to antibiotics are noted: * ($p<0.05$).

seen. After 6 h, ampicillin in combination with ultrasound produced greater than 1.5 logs of killing above killing by ampicillin alone (see Fig. 8; $p<0.0096$ after 6 h). As with the other organisms, ultrasound alone had no effect on the viability of *S. epidermidis*.

S. epidermidis treated with tetracycline. The MIC of tetracycline on *S. epidermidis* was determined to be 50 μg/ml. Experiments showed no enhanced killing with ultrasound (data not shown).

Discussion

Previous experiments have shown a synergistic effect between ultrasound and antibiotics in killing planktonic cultures of *E. coli* and *P. aeruginosa* (Pitt et al., 1994; Williams and Pitt, 1997). The purpose of this research was to determine if this same bioacoustic effect could be observed with other species of Gram-negative and Gram-positive organisms.

Ampicillin was chosen in this screening study because of its wide use against Gram-positive and Gram-negative bacteria. Previous work reported that no bioacoustic effect was observed with planktonic cultures of *S. epidermidis* and *S. aureus* at the MIC of ampicillin (Pitt et al., 1994). However, data reported here showed that a bioacoustic effect can be seen with *S. epidermidis* and *S. mitis* in combination with ampicillin at concentrations above the MIC for longer periods than when previously examined. Thus a bioacoustic enhanced killing of some Gram-positive species occurs under certain conditions.

Tetracycline was chosen for this study because of its wide use against both classes of bacteria and because of its different mode of action and uptake from those of the aminoglycosides and penicillins (Kagan, 1980; Peterson and Verhoef, 1986). The smallest degree of ultrasonic enhancement was seen with this antibiotic. Enhanced killing was seen only after extended sonication in combination with concentrations of antibiotic that were more than double the MIC. For example, *E. aerogenes* exposed to tetracycline showed significant enhanced killing only after 4 h of sonication. Tetracycline in combination with *S. derby* and *S. marcescens* showed no killing, even after extended sonication.

The aminoglycosides, gentamicin, kanamycin, and streptomycin, were also evaluated in this study (Martin and Beveridge, 1986; Tangy et al., 1985). It has been postulated that the binding of polycationic aminoglycosides to the anionic Gram-negative cell surface destabilizes the outer membrane, causing increased permeability (Kadurugamuwa et al., 1993a, b; Martin and Beveridge, 1986). Perhaps this destabilization, when combined with ultrasound, provides a mechanism for increased intracellular concentration of the antibiotic. All Gram-negative organisms we have evaluated so far, when treated with an aminoglycoside, were killed more rapidly when the antibiotic treatment was combined with 70 kHz ultrasound.

The greatest bioacoustic effect was seen with gentamicin in combination with *E. aerogenes* and *S. marcescens*. Both showed 2 to 4 logs greater killing with antibiotic and ultrasound than killing with antibiotic alone. This is similar to the ultrasonic enhanced action of gentamicin on *E. coli* and *P. aeruginosa* reported previously (Pitt et al., 1994).

Ongoing work has shown that the principle of bioacoustic-enhanced killing of bacteria also applies to sessile bacteria in biofilms (Qian, 1996; Qian et al., 1996, 1997). Although the ultrasonic-enhanced antibiotic activity in this and previous studies is beyond dispute, the molecular mechanisms producing the enhanced killing are not fully known. Electron spin resonance studies (Rapoport et al., 1997) indicate that the transport of a hydrophobic spin label either across or into the outer leaflet of the lipopolysaccharide (LPS) layer of Gram-negative organisms is enhanced by ultrasound. It is probable that the enhanced killing of the organisms tested in this study with some antibiotics is due to a similar enhanced transport of the antibiotic. A physical disruption or stress of the outer membrane through the application of ultrasound may decrease the stability of the bacterial outer membrane, thereby allowing a greater sorption or diffusion of the antibiotic. Destabilization of the outer membrane because of ultrasound alone does not kill the cells, as indicated by control experiments receiving only ultrasound. Wyber et al. (1997) demonstrated that plasmid DNA was effectively delivered into *Saccharomyces cerevisiae* by using low-frequency ultrasound. Plasmid DNA was not damaged by this method, and plasmids were transformed into *S. cerevisiae* cells more effec-

tively than with conventional methods. Using mammalian cells, Mitragotri et al. (1996) have indicated that low-frequency ultrasound destabilizes lipid layers in skin, thus enhancing the permeability of drugs. After the ultrasonic treatment, the previous permeability was eventually restored. A similar reversible destabilization could be occurring in the bacterial outer membrane.

Besides enhanced membrane permeability, there are other hypotheses regarding possible mechanisms to support this observed acoustic enhancement. One is that the physical stress caused by low-frequency ultrasound may cause a type of stress response within the cell, analogous to the heat shock response observed in both prokaryotic and eukaryotic cells. A stress response would activate specific genes coding for proteins to respond to the stress or to make the membrane more permeable to nutrients or other molecules outside the cell. This increased permeability might account for the greater uptake of antibiotics by some organisms. It is also possible that a stress response leads to increased ribosomal activity contributing to the effectiveness of drugs that bind to the ribosome.

A final hypothesis is that the low-frequency ultrasound may affect the bacteria at the ribosomal level. As mentioned above, some antibiotics act on the bacteria by binding to specific sites on the bacterial ribosome and interrupting protein transcription. Perhaps ultrasound destabilizes the binding of mRNA or the growing peptide chain to the respective grooves in the ribosome, or perhaps it prevents the attachment of amino acids to the growing peptide chain because of physical disruption. Ultrasound may destabilize the ribosome in such a way that protein synthesis is hampered entirely. These stresses coupled with an increased concentration of antibiotics (through increased membrane permeability) may account for the augmented killing of bacteria with some antibiotics. These hypotheses and others remain to be tested.

Whatever the molecular mechanism may be, these results have shown that 70 kHz ultrasound increases the killing of several species of bacteria with several classes of antibiotics. Although not all species are killed more effectively with the combination of antibiotics and ultrasound, these findings could be an important contribution to the effective treatment of some types of bacterial infections.

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